Incomplete lineage sorting and hybridization as drivers of tree discordance in *Petunia* and related genera (Petunieae, Solanaceae)

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Abstract

Despite the overarching history of species divergence, phylogenetic studies often reveal distinct topologies across regions of the genome. The sources of these gene tree discordances are variable, but incomplete lineage sorting (ILS) and hybridization are among those with the most significant biological importance. *Petunia* serves as a classic system for studying hybridization in the wild. While field studies suggest that hybridization is frequent, the extent of reticulation within *Petunia* and its closely related genera has never been examined from a phylogenetic perspective. In this study, we used transcriptomic data from 11 *Petunia*, 16 *Calibrachoa*, and 10 *Fabiana* species to illuminate the relationships between these species and investigate whether hybridization played a significant role in the diversification of the clade. We identified that tree discordance within genera can be explained by high levels of ILS due to their rapid diversification and hybridization events. Moreover, network analyses indicated hybridization between *Petunia* and *Calibrachoa*, genera that have different chromosome numbers. Although these genera cannot hybridize at the present time, ancestral hybridization could have played a role in their parallel radiations, as they share the same habitat and life history.

Introduction

Over the last two decades, systematic biology has made significant advances, managing to work with vast volumes of data and constructing robust phylogenies to elucidate species' relationships and evolutionary histories. Nevertheless, using different methods and datasets (i.e., genetic markers and sampling schemes) often results in conflicting tree topologies. These discrepancies may stem from errors in model specifications, data processing, or evolutionary processes such as incomplete lineage sorting (ILS) and hybridization (Galtier and Daubin, 2008). Coalescent-based methods are commonly employed to overcome conflicts in trees caused by ILS. However, these methods are unreliable in situations involving gene flow among lineages (Solís-Lemus et al. 2016). Thus, several methods that consider introgression have emerged, each carrying its advantages and disadvantages (Hibbins and Hahn, 2022). Despite these advances, distinguishing between these two natural phenomena remains a complex task. While high-quality genomic data and the combined use of methods relying on distinct premises are progressively improving our ability to do so (Morales-Briones et al. 2021), the power of detecting such events depends on the extent of ILS and gene flow (Kong and Kubatko, 2021).

The mounting evidence of hybridization's role in plant diversification has led botanists to recognize that evolution likely follows a web-like pattern, rather than a strictly bifurcating one (Stull et al. 2023). However, the potential outcomes of such events are highly variable (Abbott et al. 2016; Soltis and Soltis, 2009). Hybridization can facilitate speciation through novel trait combinations or polyploidization (Abbott et al. 2013), lead to extinction through genetic

swamping (Todesco et al. 2016), or introgress adaptive alleles (Suarez-Gonzalez et al. 2018). Regardless of the outcomes, hybridization is as a frequent evolutionary phenomenon at both shallow (Nevado et al. 2018) and deep timescales (Rothfels et al. 2015) with extensive impacts on plant diversification and evolution (Goulet et al. 2017; Whitney et al. 2010).

To have detectable effects on the evolutionary history of lineages, hybridization does not necessarily need to be stable and recurring over time. One admixture event is sufficient to introduce new variations into the genomes of other species or even kickstart new evolutionary lineages (Anderson, 1948; Porretta and Canestrelli, 2023). Ongoing hybrid zones are a great resource for studying introgressive hybridization, adaptation, and morphological changes in the present (Harrison and Larson, 2016). However, detecting ancient hybridization events, which might have played a role in the adaptation and establishment of older lineages, poses a greater difficulty (Stull et al. 2023). This challenge arises because it requires more sophisticated data and methods, especially due to our inability to rely on intermediate morphological phenotypes and the erosion of the genetic signatures over time (Taylor and Larson, 2019).

The *Petunia*-*Calibrachoa*-*Fabiana* Solanaceae clade presents a unique opportunity for investigating the evolutionary dynamics of plant diversification in southern South America. Notably, *Petunia* has become an important taxon to understand the role of hybridization in the region due to the multiple hybrid zones documented (e.g., Binaghi et al. 2023; Caballero-Villalobos et al. 2021; Giudicelli et al. 2019). Species from the *Petunia*-*Calibrachoa*-*Fabiana* clade have experienced rapid diversification, exhibiting a diverse range of pollination syndromes and inhabiting distinct biomes, from rainforests to savannahs and deserts (e.g. Alaria et al. 2022, Mäder and Freitas, 2019; Reck-Kortmann et al. 2014). While *Petunia* and *Calibrachoa* are very similar in morphology and ecological conditions, *Fabiana* stands out due to its drastic differences from related genera: they are xerophytic, have small flowers, and display reduced or even absent leaves (Alaria et al. 2022). The three genera are classified within the well-established tribe Petunieae, forming a strongly supported clade (Särkinen et al. 2013; Wheeler et al. 2022). However, the internal relationships within this clade remain controversial. Phylogenetic studies using distinct genetic markers, sampling schemes, and analytical approaches have produced conflicting topologies (Fig. 1). Some place *Petunia* as a sister to the remaining genera (Alaria et al. 2022; Olmstead et al. 2008; Wheeler et al. 2022), whereas others place it as a sister genus to *Calibrachoa* (Särkinen et al. 2013) or to *Fabiana* (Mäder and Freitas, 2019; Reck-Kortmann et al. 2015). These incongruences might be attributed to the substantial levels of ILS due to their rapid diversification (Särkinen et al. 2013; Wheeler et al. 2022), or potentially from instances of ancient hybridization.

The chance of hybridization depends on how effective reproductive barriers are to prevent gene flow. Barriers that prevent the formation of hybrids in the first place, i.e. prezygotic barriers, are robust mechanisms against gene flow in plants (Baack et al. 2015; Christie et al. 2022). Complete reproductive isolation typically relies on a combination of barriers. In *Petunia*, gene flow is primarily prevented by prezygotic barriers, including geographic and floral isolation, with postzygotic barriers playing a negligible role (Dell'Olivo et al. 2011). These barriers have been extensively studied in *Petunia*, which established this genus as a model in plant hybridization and pollination studies (Binaghi et al. 2023; Gübitz et al. 2009; Rodrigues et al. 2018; Turchetto et al. 2019). However, a comprehensive investigation into *Calibrachoa* and *Fabiana*, as well as the possibility of hybridization causing tree discordance among genera and congeneric species has yet to be examined under a solid phylogenetic framework.

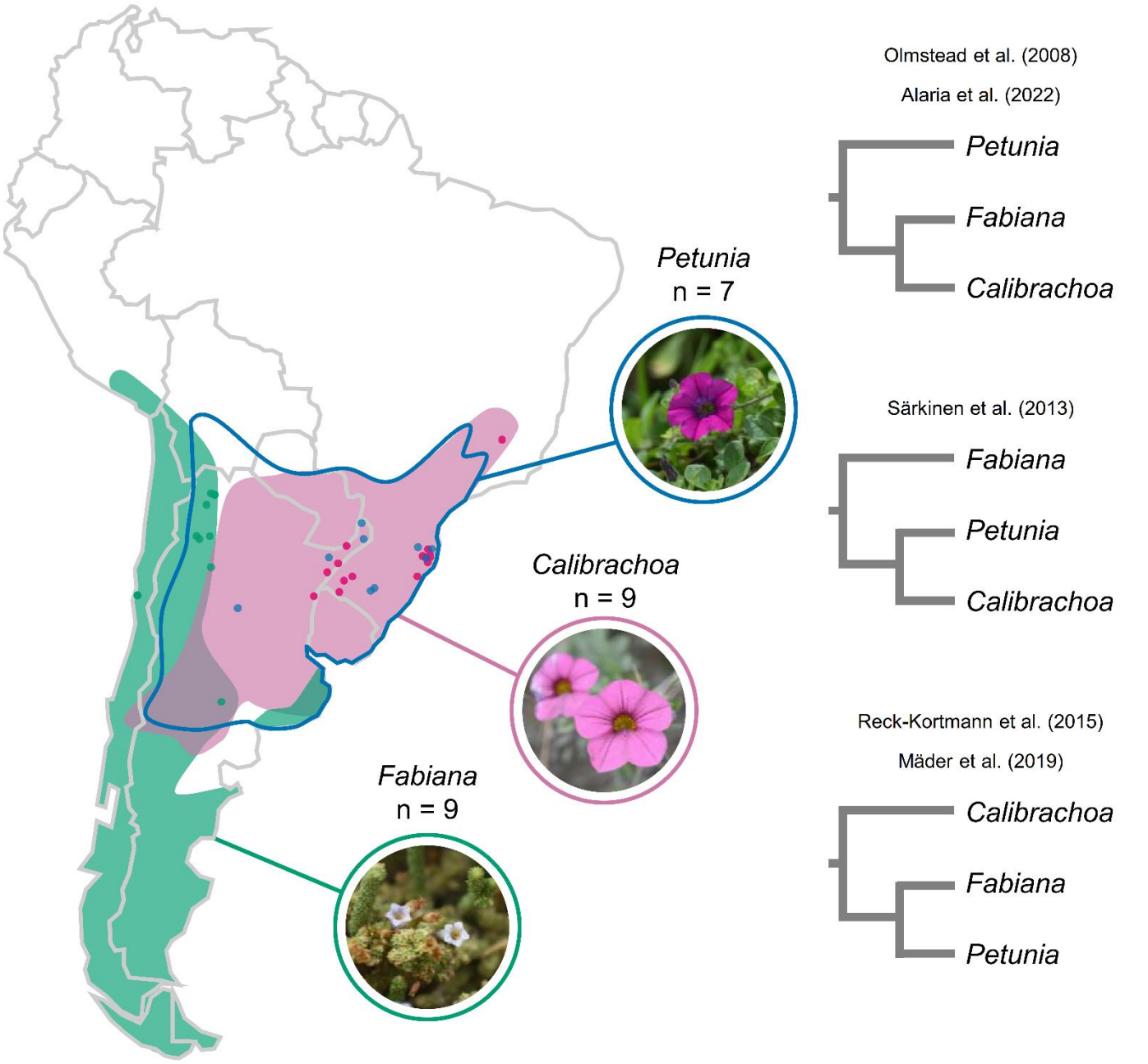

Figure 1. Distribution map of *Petunia* (blue outline), *Calibrachoa* (pink shaded area), and *Fabiana* (green shaded area) in South America. Map dots represent sampled localities, colored by genus, except for the species collected at greenhouses. Representatives of each genus are shown in circles: *Petunia altiplana*, *Calibrachoa eglandulata*, and *Fabiana bryoides* (photos: Lucas C. Wheeler). Phylogenetic relationships of the genera are presented on the right. Olmstead et al. (2008) used two plastid markers; Särkinen et al. (2013) used two nuclear and five plastid markers; Reck-Kortmann et al. (2015) used one nuclear and two plastid markers; Mäder et al. (2019) used eight nuclear and eight plastid markers; and Alaria et al. (2022) used one nuclear and three plastid markers.

Even though *Petunia* and *Calibrachoa* are similar in flower morphology, ecology, and geographic distribution (Fig. 1), they have been split into two different genera due to their chromosome numbers: *Petunia* has seven chromosome pairs (2n = 14), whereas *Calibrachoa* has nine (2n = 18) (Stehmann et al. 2009; Wijsman and De Jong, 1985). The persistence in nature of hybrids between species that have different chromosome numbers is unlikely as it leads to meiotic mispairing—unless it involves polyploidization (Alix et al. 2017; Hegarty and Hiscock, 2008). To date, polyploidization has never been observed in *Petunia* or *Calibrachoa*. Hence, the occurrence of hybrids between *Petunia* and *Calibrachoa* in the wild seems unlikely, even though some species occur in sympatry. While intergeneric hybrids known as "Petchoa" have been developed and are available commercially, these hybrids are sterile, and their creation requires significant human intervention (Shaw, 2007). In contrast, while *Calibrachoa* and *Fabiana* share the same chromosome count, which theoretically would allow successful hybrid's meiosis, their disjunct geographical distribution, and distinct life histories serve as strong barriers that prevent gene flow.

In this study, we used floral transcriptome data from *Petunia*, *Calibrachoa*, and *Fabiana* species to investigate the source of discordance among phylogenetic trees and evaluate the potential influence of reticulate evolution on the diversification of the group. Furthermore, we explored whether hybridization events are equally prevalent in *Fabiana* and *Calibrachoa* as they are in *Petunia*. We hypothesized that hybridization would occur frequently within genera, both in recent times and throughout their evolutionary history, whereas intergeneric hybridization would be unlikely due to the presence of robust reproductive barriers.

Material and Methods

Taxa sampling and transcriptome data processing

We used the raw RNA-seq data derived from Wheeler et al. (2022, 2023), focusing on the *Petunia-Calibrachoa-Fabiana* clade and incorporating six outgroup species. In total, we employed 107 individuals, encompassing 11 *Petunia* species, 16 *Calibrachoa* species, 10 *Fabiana* species, and six outgroups (Table S1). Here, we expanded the Wheeler et al. (2022) dataset by including additional individuals for most sampled species derived from Wheeler et al. (2023), resulting in three individuals per species collected at the same time and location (hereafter referred to as replicates). We corrected the raw RNA-seq reads using Rcorrector (Song and Florea, 2015) and removed adapters using Trimmomatic (Bolger et al. 2014). Subsequently, we mapped the reads against the 3,672 protein-coding genes from conspecific transcriptomes, which were assembled for replicate 1 by Wheeler et al. (2022) using BWA (Li and Durbin, 2010). Consensus fasta sequences were generated through samtools 1.16 (Li et al. 2009) by calling the most frequent base (-m simple) and then aligned with MACSE 2.06 (Ranwez et al. 2018).

Phylogenetic analyses and evaluation of tree discordance

To elucidate the phylogenetic relationships among species within the *Petunia-Calibrachoa-Fabiana* clade, we employed three distinct approaches. Firstly, we estimated the maximum likelihood (ML) gene trees using the GTR+Γ model along with 1,000 bootstrap replicates in RAxML (Stamatakis, 2014) and estimated the species tree—both with and without assigning individuals to species—using ASTRAL III 5.7.8 (Rabiee et al. 2019; Zhang et al. 2018). Secondly, we constructed a supermatrix by concatenating the fasta alignments with the *SuperMatrix* function of the evobiR R-package (Jonika et al. 2023). This supermatrix was then

used to generate a maximum likelihood species tree using IQTree 1.6.12 (Nguyen et al. 2015) setting the GTR+Γ model to each partition with 1,000 bootstrap replicates. Lastly, we estimated a species tree using SVDQuartets, a coalescent method originally designed for SNP data but also effective with multi-locus alignments (Chifman and Kubatko, 2014), implemented in PAUP* 4a (Swofford, 2003), which infers relationships among quartets and subsequently summarizes these relationships into a species tree. We set the outgroups, assigned individuals to respective species, and assessed all quartets (*evalq=all*) using 200 multi-locus bootstrap replicates.

We used phyparts (Smith et al. 2015) to evaluate the number of concordant and conflicting bipartitions among gene trees in comparison to the inferred ASTRAL species tree setting support level of at least 50% for the corresponding node (-*s* 0.5). Due to computational limitations and the observed clustering of conspecific individuals (see Results), we pruned replicates 2 and 3 for all species using Newick utilities (Junier and Zdobnov, 2010). Since phyparts requires rooted trees as input, we set *Bouchetia erecta* as the root, which led to a dataset of 3,471 gene trees where the outgroup was present. For the ML phylogenetic tree, we evaluated genealogical concordance with gene concordance factor (gCF) and site concordance factor (sCF) with 100 randomly sampled quartets (-*scf*), where gCF measures how often a specific branch in the species tree is supported by "decisive" gene trees, while sCF measures the percentage of sites that support a branch in the tree (Minh et al. 2020).

Detection of hybridization

We used HyDe (Blischak et al. 2018) to search for hybridization signals. HyDe relies on phylogenetic invariants to estimate admixture (γ), where a γ value of 0.5 signifies an equal genetic contribution from each parental species, and values approaching 0 or 1 indicate a greater genetic contribution of one of the parental species. We used a concatenated matrix of alignments and trimmed sites with trimAl (Capella-Gutiérrez et al. 2009) with options -*gt* 0.5 and -*cons* 60, which yielded 5,209,834 sites. We assigned individuals to species and set the six outgroup species as outgroups, which resulted in an evaluation of 23,310 triplets. As a second approach, we employed QuIBL (Edelman et al. 2019) which relies on branch lengths of gene trees to assess whether hybridization provides a more plausible explanation for the divergence patterns compared to ILS alone. Because QuIBL requires that all taxa be present in every gene tree, we created a dataset with no missing loci for all ingroup species and *B. erecta*. All trees were rooted in *B. erecta* and pruned to contain only one individual of each species with Newick Utilities, which resulted in a final dataset of 826 gene trees.

Reticulate evolution and network reconstruction

Considering the possibility of a non-bifurcating evolutionary history of the *Petunia-Calibrachoa-Fabiana*, we inferred phylogenetic networks that account for both ILS and gene flow among taxa. Due to computational limitations, we constructed a reduced dataset comprising 18 ingroup taxa and a single outgroup species (Table S1), not allowing for missing loci, which resulted in a dataset of 1,215 loci. We estimated gene trees with RAxML and the species tree with ASTRAL as described in the previous section.

As our first approach, we estimated a phylogenetic network with the maximum pseudolikelihood method SNaQ implemented in the Julia package PhyloNetworks 0.16.2 (Solís-Lemus et al. 2017; Solís-Lemus and Ané, 2016). We searched for up to five hybridization events ($h = 5$) and used the ASTRAL phylogeny as the starting tree. For the following steps, we used the network from the previous estimation as the starting network. The best number of

hybridization events was selected based on where we could detect a steep log-pseudolikelihood improvement. After selecting the best number of hybridization events, we ran 100 bootstrap replicates using the 1,000 bootstrap ML gene trees inferred by RAxML with default settings.

As a second approach, we estimated a network with the command "InferNetwork_MPL" in PhyloNet 3.8.2 (Than et al. 2008), also searching for up to five hybridization events and 10 runs for each search. To select the best-scored network, we used the "CalGTProb" function in PhyloNet (Yu et al. 2012) to get network likelihoods. We compared the networks with model selection using the Akaike information criterion (AIC; Akaike 1973), the bias-corrected Akaike information criterion (AICc; Sugiura 1978), and the Bayesian information criterion (BIC; Schwarz 1978). We set the number of parameters to the number of estimated branch lengths and hybridization probabilities, correcting for finite sample size with the number of gene trees used.

Considering the potential occurrence of intergeneric hybridization (see Results), we used Twisst (Martin and Van Belleghem, 2017) on the reduced dataset of 19 species and 1,215 loci. We categorized species according to their respective genera and designated *B. erecta* as the outgroup, resulting in three potential topologies. We computed the topology weight and determined the frequency of specific topologies within the gene tree set, that is, we counted the number of trees supporting one of the three possible topologies. Subsequently, we conducted a chi-square test to compare the occurrences of the two minor topologies (Owens et al. 2023; Suvorov et al. 2022). The null hypothesis stated that, in the case of ILS alone, each of these minor topologies should account for 50% of the occurrences.

Results

Phylogenetic relationships and tree discordance within the Petunia-Calibrachoa-Fabiana clade

The gene count for each replicate ranged from 2,937 to 3,573 (Table S1), and the final concatenated matrix consisted of 5,687,285 base pairs. The resulting phylogenetic trees constructed using multiple methods consistently positioned *Petunia* as sister to *Calibrachoa* + *Fabiana* (Olmstead et al. 2008; Alaria et al. 2022; Fig. 1), while revealing discordant intrageneric topologies. Both the supertree (ASTRAL) and the supermatrix strategies (IQTree) exhibited strong support for most branches (LPP = 0.95–1 in ASTRAL and bootstrap = 100 in IQTree; Fig. 2). Nonetheless, the two methods estimated different relationships for multiple branches within *Petunia* and within *Calibrachoa*, which might be expected given the high proportion of conflict among gene trees apparent from the phyparts analysis (Fig. 2). We did, however, find that the replicates from a single species consistently group together in the ASTRAL analysis with robust support (Fig. S1), supporting assignment of individuals to species. As might be expected given the differences between the ASTRAL and supermatrix trees (Fig. 2), SVDQuartets displayed high support for deeper nodes, but weaker support for shallower nodes within *Calibrachoa* and *Petunia* (Fig. S2), indicating extensive ILS and possibly intrageneric hybridization. IQTree Concordance Factor results indicated that the gCF values were notably low for shallow nodes, whereas sCF values offered greater support for these relationships than gCF, suggesting that genetic sites were more consistent in inferring evolutionary relationships at these shallower nodes than the genes themselves (Fig. 2).

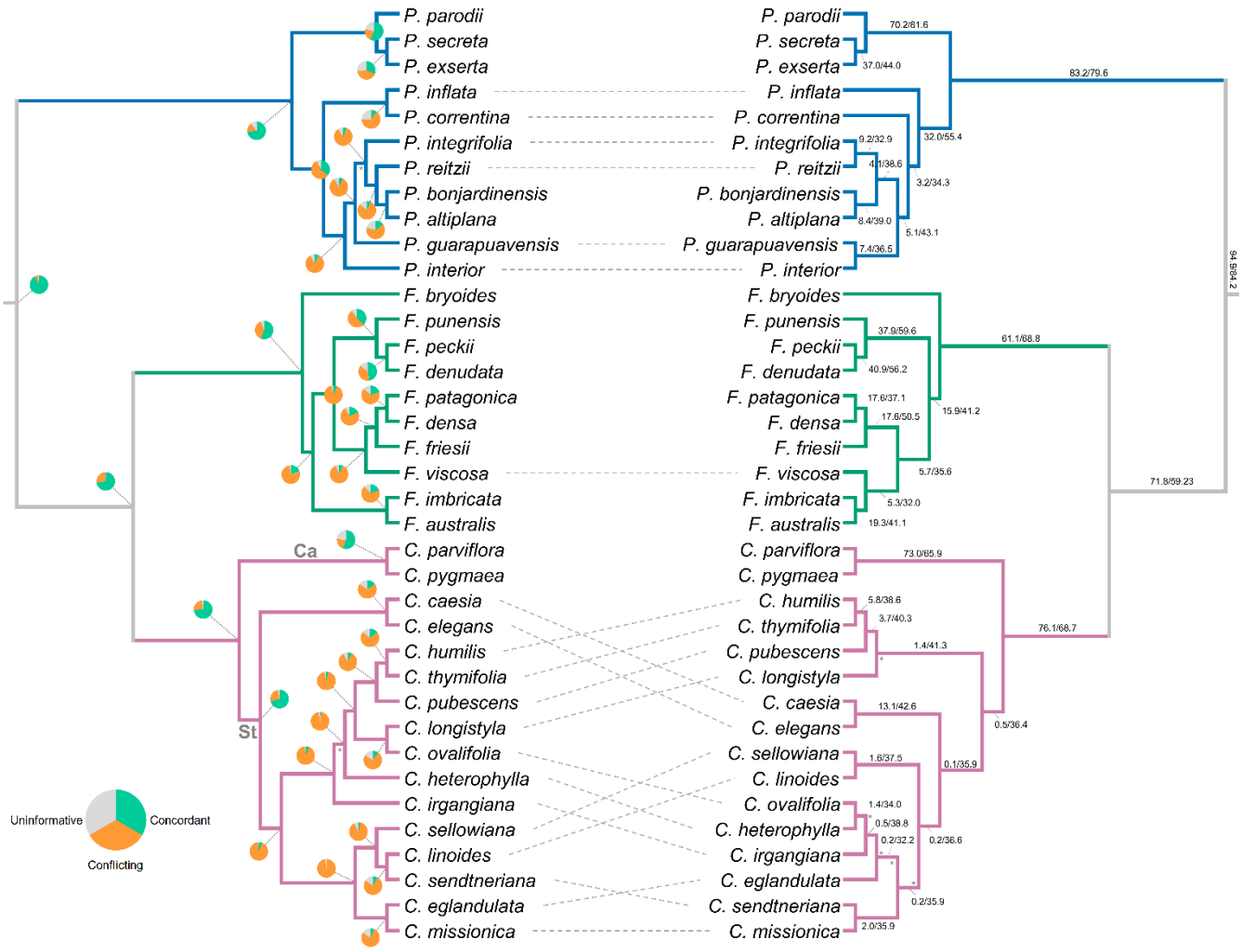

Figure 2. Phylogenetic trees of the *Petunia-Calibrachoa-Fabiana* clade inferred from ASTRAL (left) and IQTree (right). The subgenera of *Calibrachoa* are annotated on their branches on ASTRAL tree: Ca for *Calibrachoa*, and St for *Stimomphis*. All nodes are strongly supported (local posterior probability=1 for ASTRAL/boostrap=100 for maximum likelihood tree), except when otherwise noted by an asterisk (*). Dashed lines represent species with differing positions in the two trees. Pie charts on the ASTRAL tree depict gene support based on 3,471 gene trees: corroborating (green), conflicting (orange), or uninformative (gray; < 50% bootstrap scores or missing loci) relationships. The numbers above the branches on the maximum likelihood tree inferred from IQTreethe indicate gene and site Concordance Factors (gCF and sCF, respectively). Outgroup species are not shown for simplicity.

Reticulate evolution and species networks

The search for hybrids resulted in several significant hybrid triplets, both in QuIBL and HyDe (Fig. 3). HyDe resulted in 3,352 significant triplets, even between intergeneric species (Table S2). However, when we only considered events with $0.2 < \gamma < 0.8$ (to detect more recent hybridization events, where we can detect greater parental contribution from both species, and discard spurious results with low contribution from either parent), these hybridization events were constrained within genera (Fig. 3). QuIBL showed several minor topologies that could not be explained by ILS alone, although the percentage of discordant loci explained by introgression were lower than 10% in all cases (Fig. S3; Table S3).

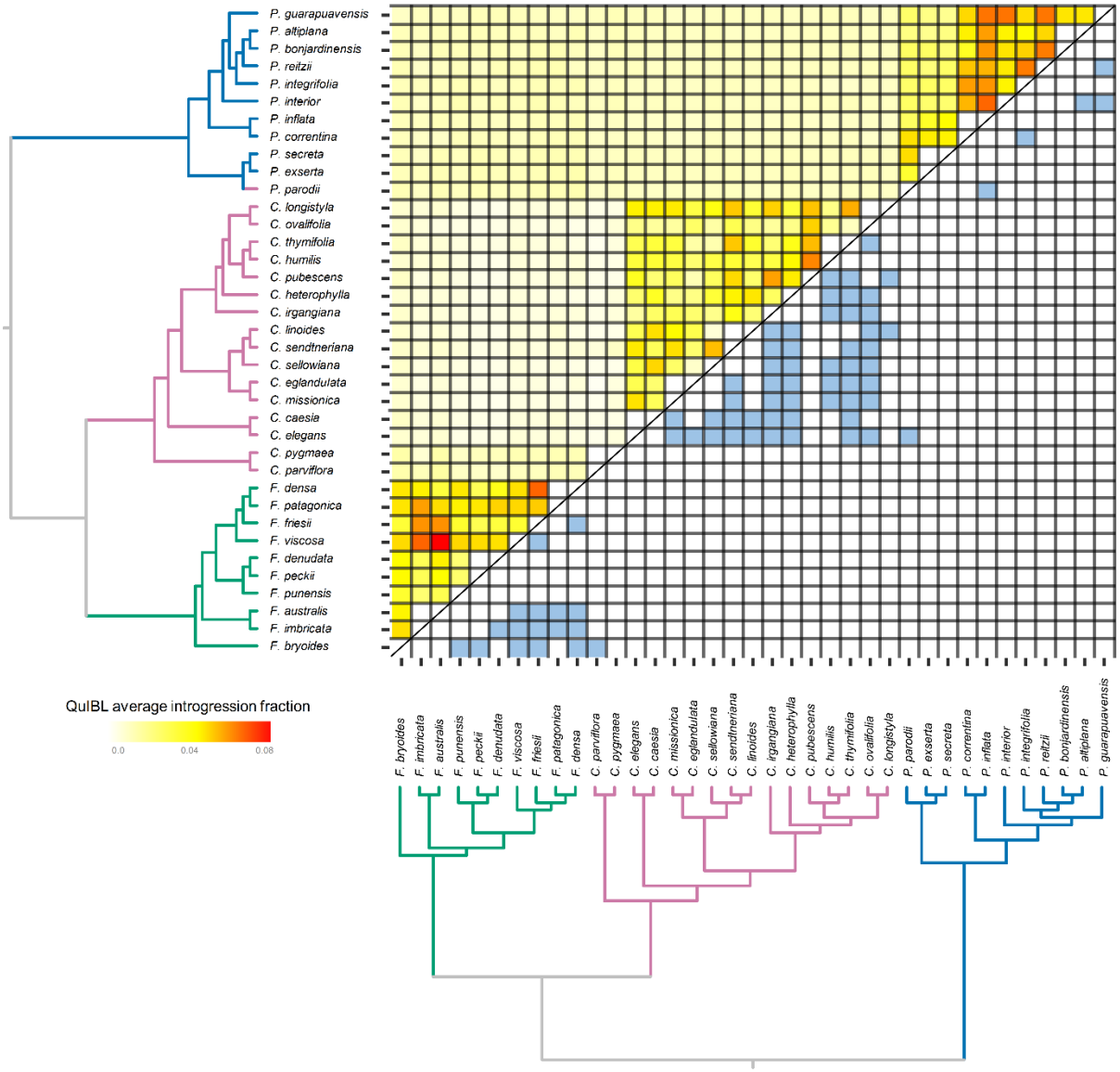

Figure 3. Detected hybridization events using QuIBL (top) and HyDe (bottom), using ASTRAL phylogeny as the reference species tree. The HyDe graph displays hybridization events with $0.2 < \gamma < 0.8$; refer to Table S2 for complete results. QuIBL identified introgression events based on branch lengths in 826 gene trees with single individuals from each taxon; refer to Table S3 for complete results.

Although network inferences yielded different optimal numbers of reticulations (one in SNaQ, two in PhyloNet; Fig. 4), both agreed on an ancient hybridization edge from *Petunia* to *Calibrachoa* subgenus *Stimomphis*. However, the inheritance probabilities for this introgression were low in both analyses (less than 1% in SNaQ and 3.4% in PhyloNet). The bootstrap analyses for SNaQ showed high support for the species network nodes, but low support for the hybrid edge. The placement of minor and major edges was not consistent, with low consistency for both the origin and the source of hybridization. The addition of more hybridization events in SNaQ usually led to the impossibility of rooting the tree in the outgroup (supplemental material online), which suggests incorrect placement of that hybridization edge.

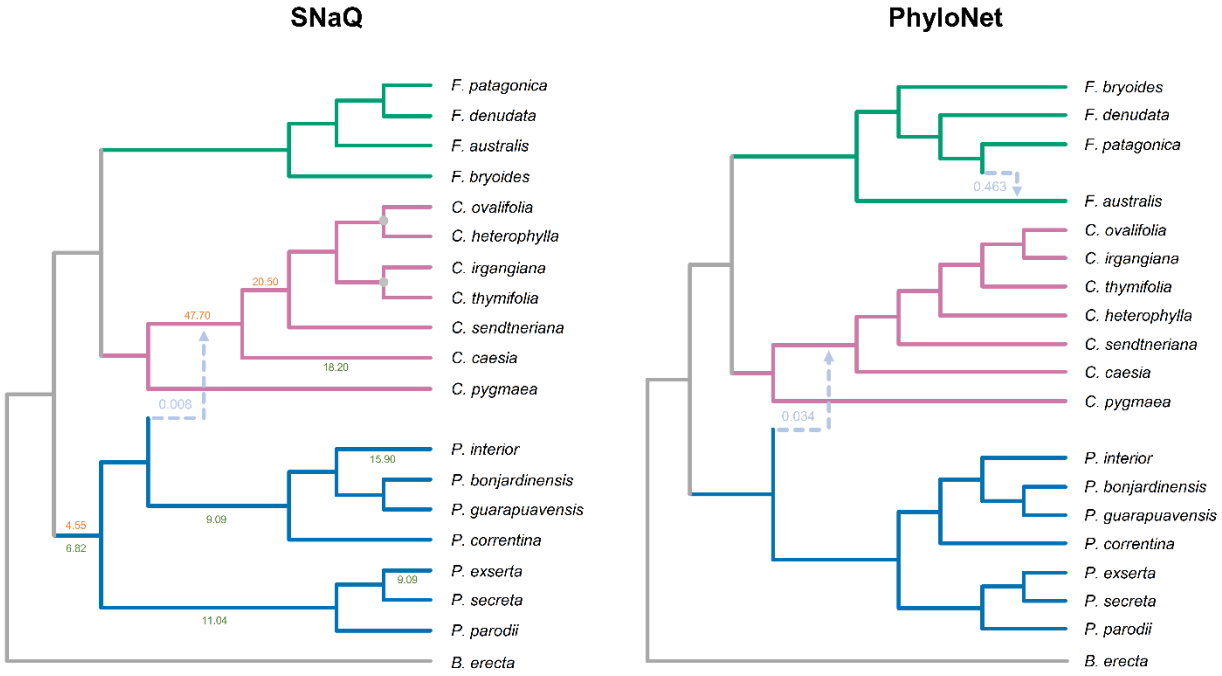

Figure 4. Inferred species networks using SNaQ (left) and PhyloNet (right) based on a reduced dataset comprising 18 ingroup species and 1,125 genes, rooted in *B. erecta*. The SNaQ tree identified the optimal network with one hybridization event. All branches received 100% bootstrap support from 100 replicates, except those signed with gray circles. The dashed line represents the minor edge, displaying the inheritance probability of the best network. The bootstrap values for minor (origin) and major (source) edges of alternative networks are colored green and orange, respectively. The PhyloNet network indicated the optimal network with two hybridization events. The dashed lines also indicate the minor edges with corresponding inheritance probabilities.

The model selection for PhyloNet revealed the network with two hybridization edges as the optimal network and highlighted that any species network is better fitting than the bifurcating species tree (Table 1). In addition to the intergeneric hybridization edge, PhyloNet also suggested a hybridization within *Fabiana* as a second hybridization event. In this case, it showed a high inheritance probability of 0.46 from *F. patagonica* to *F. australis*.

Table 1. Network likelihoods derived from the reduced dataset using PhyloNet. The number of parameters (k) represents the number of estimated branch lengths and admixture probabilities. The optimal network is in bold.

Topology	Maximum number of reticulations	Number of inferred reticulations	Total log probability	lnL	k	AIC	ΔAIC	AICc	BIC
Astral	0	NA	-	-30833.49	37	61740.98	4676.06	61743.37	61781.11
Network 0	0	NA	-389607.21	-29310.04	37	58694.08	1629.16	58620.08	58734.21
Network 1	1	1	-388824.25	-28520.99	39	57119.98	55.06	57041.98	57162.28
Network 2	**2**	**2**	**-388777.78**	**-28491.46**	**41**	**57064.92**	**0.00**	**56982.92**	**57109.39**
Network 3	3	3	-388791.27	-28511.48	43	57108.96	44.04	57022.96	57155.60
Network 4	4	3	-388805.06	-28524.33	43	57138.66	73.74	57048.66	57181.30
Network 5	5	3	-388827.51	-28543.19	43	57180.38	115.46	57086.38	57219.02

Results from Twisst revealed that, between the two minor topologies, the topology positioning *Fabiana* as the sister group to *Petunia* and *Calibrachoa* exhibited a marginally greater frequency (Fig. 5), although this difference was not statistically significant (chi-square = 1.15, *P* value = 0.28). Thus, the difference in the number of topologies of gene trees is primarily attributed to ILS and not gene flow.

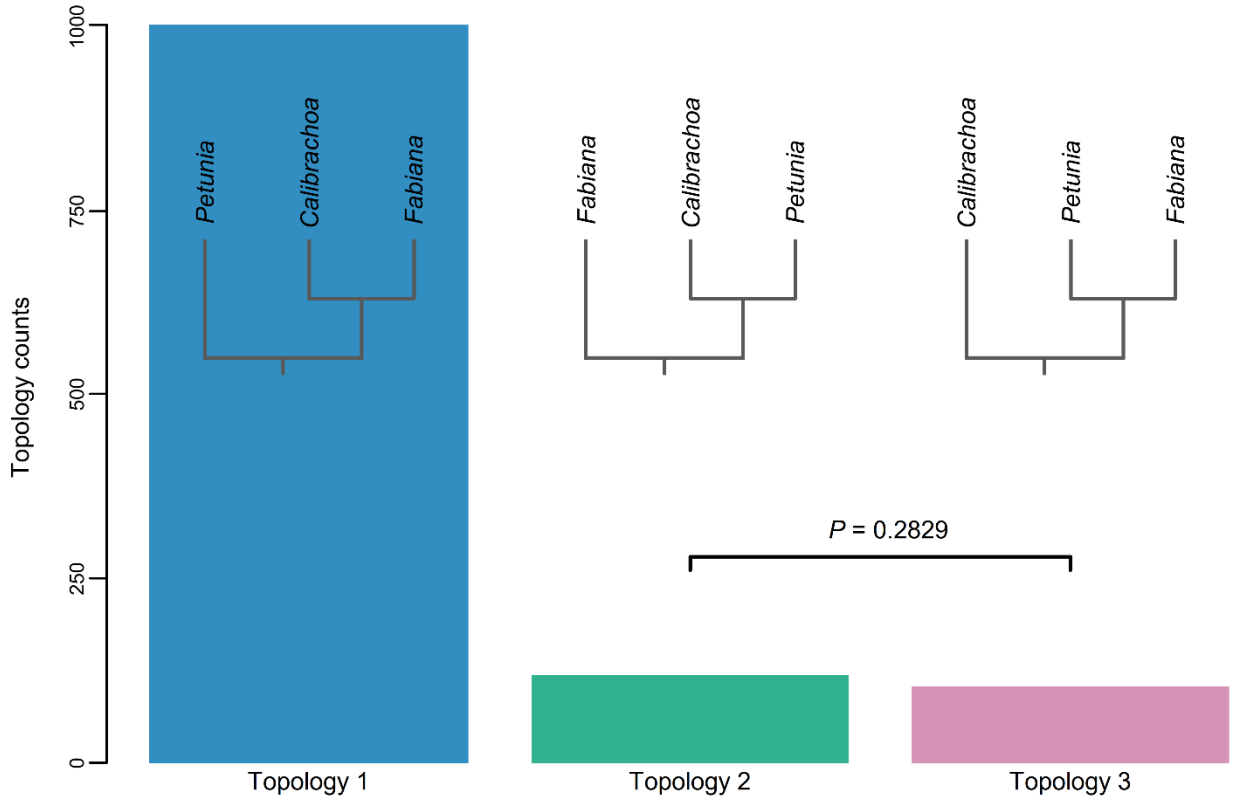

Figure 5. Total count of topologies by Twisst for the subset of 1,215 gene trees. The *P* value indicates the lack of significance for the chi-square test between the two minor topologies that places *Calibrachoa* or *Fabiana* as the outgroup.

Discussion

Extensive ILS and ancient hybridization are the sources of tree discordance in the Petunia-Calibrachoa-Fabiana clade

Historically, phylogenies based on Sanger sequences often yielded conflicting species trees among *Petunia*, *Calibrachoa*, and *Fabiana*. However, our results consistently placed *Petunia* as a sister group to *Calibrachoa* + *Fabiana*, mirroring previous findings by Olmstead et al. (2008), Alaria et al. (2022), and Wheeler et al. (2022). These results are surprising due to the morphological and ecological similarities between *Petunia* and *Calibrachoa*, suggesting that bee-pollinated herbs (such as most extant *Calibrachoa* and *Petunia* species) represent the ancestral state, and that the extreme xerophyte traits found in *Fabiana* (tiny flowers and reduced leaves) are derived features. Moreover, the more arid and temperate range of the clade likely represents a southward expansion from the shared distribution of *Petunia* and *Calibrachoa*, both of which likely originated in the lowland grasslands of southern Brazil, Uruguay, and northeast Argentina (Reck-Kortmann et al. 2014; 2015; Mäder and Freitas, 2019). Nonetheless, the relationships within each genus remained inconsistent, with high levels of conflict among all reconstructed trees. Moreover, we found extensive gene flow within genera, as supported by both QuIBL and HyDe (Fig. 3) analyses.

Our results corroborated the subdivision of *Petunia* into two main clades, the long corolla tube and the short corolla tube clades (Reck-Kortmann et al. 2014). The former is characterized by a wide range of flower colors and pollinators, represented here by *P. axillaris* subsp. *parodii* (white, hawkmoth-pollinated), *P. exserta* (red, hummingbird-pollinated), and *P. secreta* (purple, bee-pollinated). In contrast, the latter consists of species with purple flowers primarily pollinated by bees, represented here by the remaining seven *Petunia* species. The long corolla tube clade is noteworthy for its documented history of extensive hybridization (e.g., Caballero-Villalobos et al. 2021; Giudicelli et al. 2019), whereas records of interspecific hybridization within the short corolla tube clade are rare and, until now, limited to *P. interior* and *P. inflata* (Pezzi et al. 2022).

The short corolla tube clade displayed a higher number of discordant gene trees and shorter branch lengths compared to the long corolla tube clade, indicative of rapid radiation. These species are often rare and endemic (Souza et al. 2022), occurring primarily in southern Brazil. Geographic isolation serves as the primary reproductive barrier among these species, and pollinators do not impose any reproductive barriers because they are shared among species (Stehmann et al. 2009). Despite their geographic isolation due to microhabitat adaptation, many of these species have overlapping distributions, and all are self-incompatible (Stehmann et al. 2009). This scenario presents an opportunity for interspecific gene flow. Whereas field observations have documented only a few hybrids, the substantial level of polymorphism shared between these species could be attributed to high levels of ILS (Lorenz-Lemke et al. 2010) or ongoing and recent hybridization events (Fig. 3).

Calibrachoa is classified into two subgenera: *Calibrachoa* and *Stimomphis*. The subgenus *Calibrachoa* comprises just two species that exhibit significant differences in reproductive biology and habitat compared to species in the subgenus *Stimomphis* (Fregonezi et al. 2013). Intriguingly, no instances of hybridization have been observed between *Calibrachoa* and *Stimomphis* species, underscoring the presence of robust reproductive barriers between subgenera. *Stimomphis* showed a similar evolutionary history to the one observed in the short corolla *Petunia* clade: rapid radiation, high levels of conflicting gene trees, and extensive ILS. The topology recovered here exhibits minimal congruence with prior studies (Fregonezi et al. 2012, 2013; Mäder and Freitas, 2019). Notably, the highland clade identified by Mäder and

Freitas (2019), represented here by *C. elegans*, *C. eglandulata*, *C. sendtneriana*, and *C. linoides*, did not emerge in any of our phylogenetic reconstructions. Such high levels of conflict among phylogenetic methods are expected with extensive ILS and hybridization. Moreover, it is worth noting that, except for *C. parviflora*, all these species are self-incompatible (Fregonezi et al. 2013), facilitating the potential for hybridization as these species cannot prevent heterospecific gene flow through autonomous selfing (Brys et al. 2015).

It has been demonstrated that ML concatenation methods are often inadequate for accurately recovering species trees when extensive ILS is involved (Kubatko and Degnan, 2007; Mendes and Hahn, 2018). Therefore, in scenarios such as the short corolla *Petunia* clade and *Calibrachoa* subgenus *Stimomphis*, ASTRAL is a more suitable alternative. These clades display extremely short branch lengths, suggesting they went through a rapid diversification process, not allowing for genes to coalesce. Species that underwent rapid radiation tend to fall in the "anomaly zone", where the most frequent gene trees do not align with the species tree (Degnan and Rosenberg, 2006; Linkem et al. 2016) which could explain the high levels of tree discordance observed here. To address this challenge, one potential strategy is to sample multiple individuals from the same species (Degnan and Rosenberg, 2006), as was done here. However, it is important to note that our individuals were sampled on the same site (Table S1), which might not provide sufficient resolution. However, when investigating clades harboring numerous rare and endemic species, sampling from the same locality is often unavoidable, but it still provides valuable biological insights.

Among the three genera, *Fabiana* exhibited a lower level of tree discordance and greater consistency among phylogenies. The previously available *Fabiana* phylogeny included only eight species but agreed on the close relationship between *F. imbricata* and *F. australis* (Alaria et al. 2022). Here, *F. viscosa* displayed varied phylogenetic placement and the highest level of introgression in QuIBL analyses, indicating significant gene flow, particularly with *F. australis* (Fig. 3). This implies that, in addition to ILS, hybridization plays a central role in causing tree discordance for these species. Intriguingly, these two species do not currently occur in sympatry.

It is crucial to recognize that methods that rely on summary statistics of triplets or quartets, such as HyDe, are highly sensitive to substitution rate variation across lineages and genes (Frankel and Ané, 2023), resulting in a high rate of false positives. In addition, such methods can fail to identify hybridization involving ghost or unsampled taxa (Bjorner et al. 2022), and they often struggle to discern gene flow between sister species, where allele sharing could be interpreted as ancestral polymorphism (Mallet et al. 2016). Branch length methods are also susceptible to the influence of rate variation. For instance, QuIBL has demonstrated a propensity for producing false positives when using shorter alignments in comparison to alignments longer than 1,000 bp (Koppetsch et al. 2023). Notably, our QuIBL dataset exhibited a median length of 494 bp, with10% of the alignments being longer than 900 bp. The *Stimomphis* species and *Petunia* short corolla clade diverged recently and are probably exempt from this rate variation; thus, the intrageneric hybridization events detected by both HyDe and QuIBL are likely authentic. However, we should interpret these results with caution as there is still potential for the rate variation assumption to be violated.

Would it be possible for Petunia and Calibrachoa to hybridize?

Our network analyses suggested an introgression event from *Petunia* to *Calibrachoa* subgenus *Stimomphis*, which given recent dating estimates (Särkinen et al. 2013) would have occurred roughly 8mya. These two genera differ in their chromosome number, with *Petunia* having a

haploid chromosome number of seven (Stehmann et al. 2009) and *Calibrachoa* having nine (Wijsman and De Jong, 1985), as in *Fabiana* (Acosta et al. 2006). Such differences in chromosome numbers typically impose a strong postzygotic barrier against hybridization, either preventing it entirely or resulting in hybrid sterility (Levin, 2002). Nevertheless, instances of hybridization and introgression between plant species with different ploidy numbers have been documented (Chapman and Abbott, 2010) and both *Petunia* and *Calibrachoa* subgenus *Stimomphis* share similar geographic distribution, morphology, habitat, and potential group of pollinators (Stemann et al. 2009). One possible explanation for our result is that this change in chromosome number occurred in the ancestral lineage of *Petunia* after the admixture event, such as in the scenario posited by PhyloNet, where the admixture event is from the common ancestor of all *Petunia* species. However, SNaQ contradicts this hypothesis, as the introgression event is inferred to have taken place after reduction in chromosome number in the common ancestor of *Petunia* (Fig. 4). Alternatively, during diversification, sufficient chromosomal homology may have enabled meiotic pairing. Artificial crosses between *Calibrachoa* and *Petunia* demonstrated some success in embryo formation but failed in germinations (Olschowski et al. 2013). However, in a recent study, Milicia et al. (2021) crossed *P. inflata* with *C. hybrida*. Despite a significantly lower percentage of viable pollen granules compared to intrageneric crosses, the hybrids produced 5% of viable pollen, highlighting the flexibility plant species have in chromosome rearrangement to allow successful meiosis. Thus, hybridization between these genera may not be out of the question. Additionally, the low chance of current hybridization does not exclude the possibility of ancient hybridization.

Despite the inference of intergeneric hybridization from both SNaQ and PhyloNet, we did not detect any support for such an event from out Twisst analysis. Instead, the discordance appears best explained by ILS as the two minor topologies are present in nearly equal frequencies (Fig. 5) We note that inheritance probabilities from SNaQ and PhyloNet were very low (1 to 3%, Fig. 4), and thus, this reticulation event, if it occurred, might be at the boundary of detection. Regardless, introgression of even a small fraction of the genome could potentially carry a large phenotypic effect (Clarkson et al. 2014), and merits future investigation when full genomes become available for these genera (Bombarely et al. 2010).

Conclusions

Here, we investigated the origins of tree discordance in the *Petunia-Calibrachoa-Fabiana* Solanaceae clade using a comprehensive genome-scale dataset encompassing multiple species. Our results confirm *Petunia* as the sister genus to *Calibrachoa* + *Fabiana*. However, the relationships among species within these genera remain unsolved. The discordance in tree topologies within the short corolla tube *Petunia* clade and *Calibrachoa* subgenus *Stimomphis* arises from a combination of ILS due to their rapid diversification and past and ongoing hybridization events. Instances of high ILS and extensive hybridization are not uncommon in the evolutionary history of plants (e.g., Kleinkopf et al. 2019; Morales-Briones et al. 2021; McLay et al. 2023), but pinpointing the specific taxa involved in the hybridization events is still a daunting task, and one of the reasons why different methods often yield conflicting results (Gates et al. 2023). These introgression events likely contributed to the species' genetic diversity, aiding their adaptation during their radiation. Additionally, our network reconstructions indicated potential intergeneric hybridization between *Calibrachoa* and *Petunia*, two genera characterized by distinct chromosome numbers. Considering the weak hybridization signals observed in network analyses, the lack of support from gene tree topology weights, and the known current barriers

due to differing base chromosome numbers, it leads us to believe that such a hybridization event did not occur. However, both of our network analyses indicated intergeneric gene flow, suggesting there is still a remote possibility that this could have occurred and may have been facilitated by strong selection despite the barrier imposed by differing chromosome base numbers between the two genera. Whole genome analyses could solve the intergeneric hybridization puzzle and contribute to ascertaining which genomic regions may have been involved in the *Petunia*-*Calibrachoa* introgression. Overall, our study sheds light on the complex evolutionary history of this charismatic South American clade, providing crucial insights into its adaptation and diversification.

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Data availability

All scripts and processed data files are available at https://github.com/pedrohpezzi/Petunia-Calibrachoa-Fabiana_TreeDiscordance.git and https://figshare.com/s/c3f6e7305660e03031ec. The raw RNA-seq data files are available in SRA under the BioProject accession number PRJNA746328.

CRediT author contributions

Pedro H. Pezzi: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Lucas C. Wheeler:** Conceptualization, Data Curation, Methodology, Software, Investigation, Writing - Review and Editing. **Loreta B. Freitas:** Conceptualization, Writing - Review and Editing, Investigation, Supervision. **Stacey D. Smith:** Conceptualization, Resources, Investigation, Writing - Review and Editing, Supervision, Project administration, Funding acquisition.

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Supplemental figures

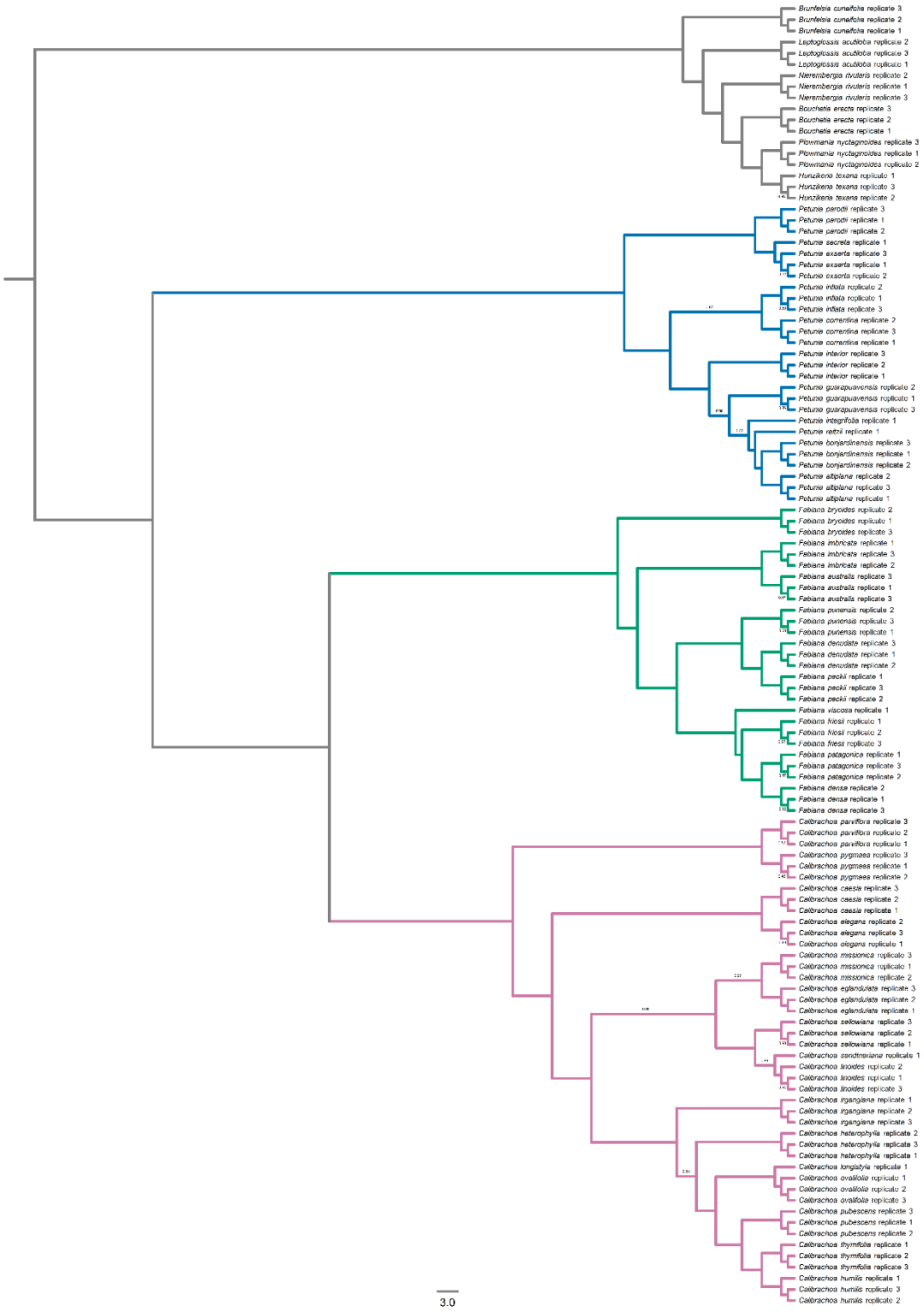

Figure S1. ASTRAL phylogenetic tree depicting individuals without species assignments. Numerical values on branches indicate local posterior probabilities that are below 100. The scale bar denotes branch length in coalescent units.

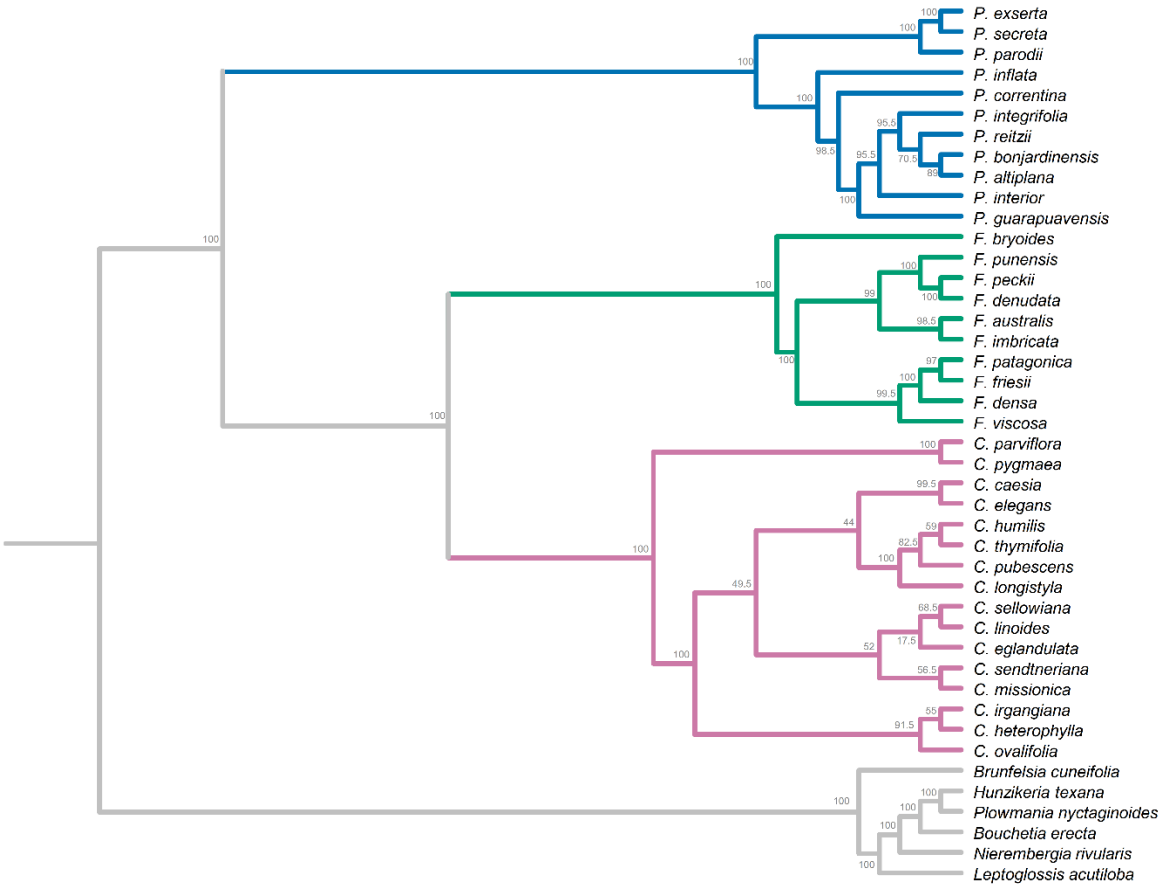

Figure S2. Phylogenetic tree depicting the relationship within the *Petunia-Calibrachoa-Fabiana* clade and the outgroups. The tree was constructed using quartets of taxa and the coalescent model with SVDQuartets. Branch numbers indicate nonparametric bootstrap values, derived from 200 bootstrap replicates.

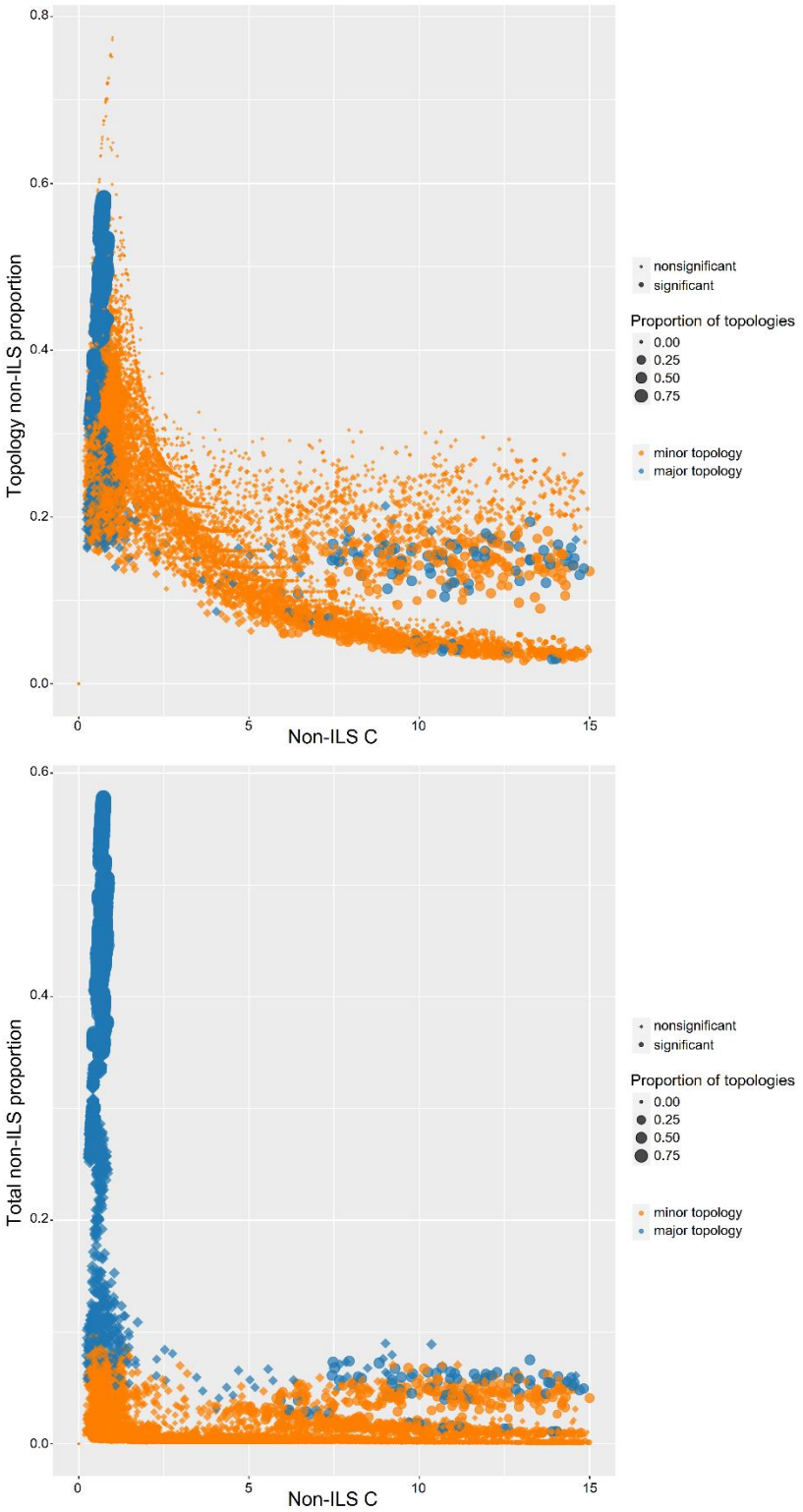

Figure S3. Summary Graphs of QuIBL Results. The upper graph illustrates the proportion of topologies not solely explained by Incomplete Lineage Sorting (ILS), while the lower graph depicts the proportion of gene trees indicating introgression events.

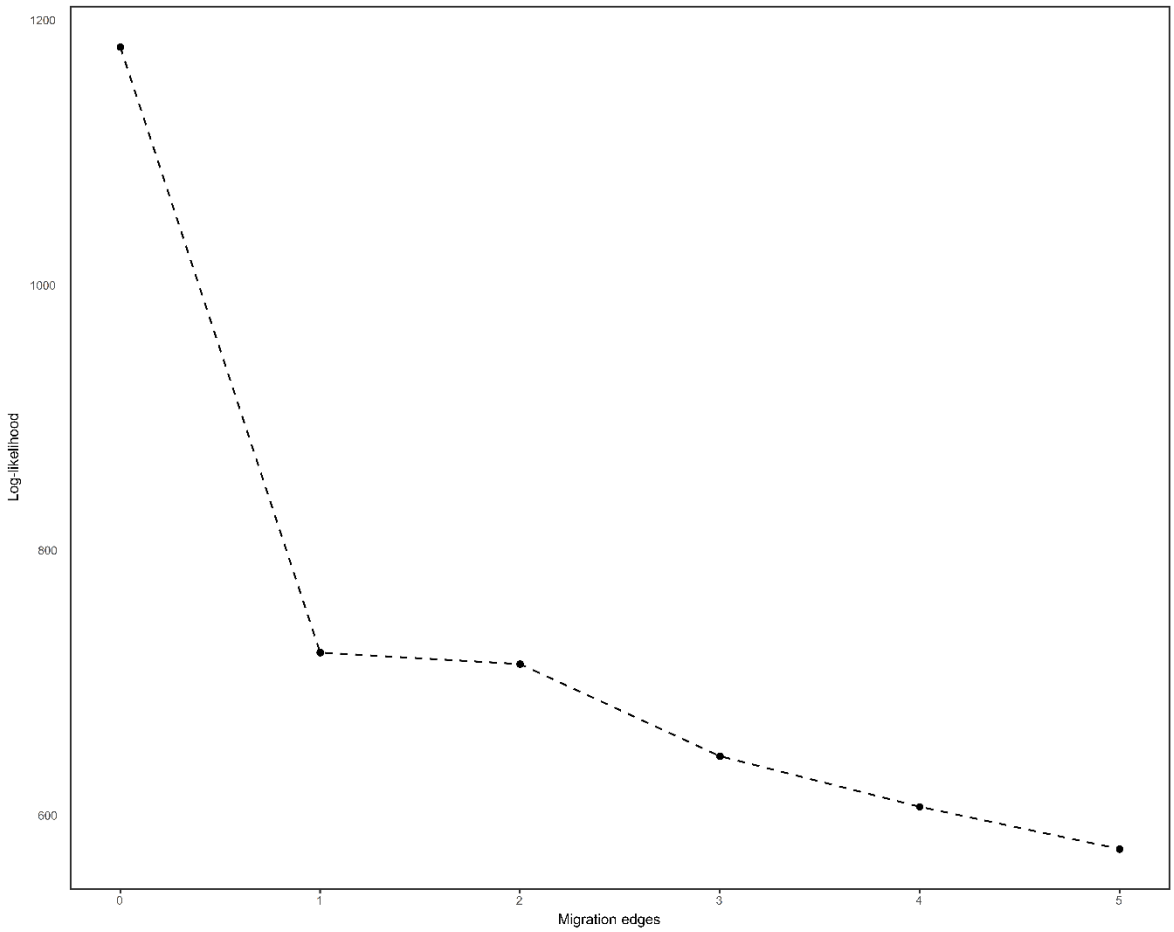

Figure S4. Log-likelihood results from the SNaQ analysis. The steepest change in log-likelihood signifies the optimal network with a single reticulation.

Supplemental tables are available on GitHub.